

SPECIFICATION

Drug Composition Containing A Lecithin-modified
Superoxide Dismutase

Technical Field

The present invention relates to a drug composition containing a lecithin-modified superoxide dismutase (hereinafter, sometimes referred to as simply PC-SOD) and a drug carrier, particularly sucrose, and to a treatment agent for diseases composed of them. The present invention also relates to an agent and a method which inhibit reduction of PC-SOD activity and the like through using sucrose as an effective ingredient.

Background Art

Prior arts closest to the present invention will be described.

- (a) PC-SOD usable in the present invention and a drug containing this as an active ingredient are described in Japanese Patent Laid-Open No. 9-117279.
- (b) A biologically active protein composition for oral and topical administrations containing a lecithin-modified biologically active protein is described in Japanese Patent Laid-Open No. 3-170438. Superoxide dismutase (hereinafter, sometimes referred to as simply SOD) is described as an example

of the biologically active protein and sugar is exemplified as an additive.

However, in these prior arts, any specific combination for a drug composition containing PC-SOD and sucrose is not described. In addition, there were no solutions for problems including: a reduction of activity of PC-SOD due to long term storage; property when lyophilized; and appearances of peaks of analogues when analyzed by column chromatography.

Disclosure of the Invention

An object of the present invention is to provide a drug composition containing PC-SOD with a less reduction of PC-SOD activity (i.e. higher stability) due to long term storage; good property when lyophilized; and controlled appearances of peaks of analogues when analyzed by column chromatography.

The present inventors examined assiduously about a drug composition having PC-SOD as an active ingredient in order to solve the above described problems, and consequently found that: a drug carrier, particularly sucrose, inhibits a reduction of PC-SOD activity due to long term storage (maintains stability); retains good property when lyophilized; has an action of controlling appearances of peaks of analogues when analyzed by column chromatography; and that this enables to provide a drug composition which can solve the above problems, leading completion of the present invention.

That is, the present invention provides a drug composition (hereinafter, referred to as the drug composition of the present invention) characterized by including PC-SOD represented by the following general formula (I) and a drug carrier, particularly sucrose:



(wherein SOD' is a residue of superoxide dismutase; Q is a chemical crosslinking; B is a residue without a hydrogen atom of a hydroxyl group of lysolecithin having the hydroxyl group at the 2-position of glycerol; m is an average number of bonds of lysolecithin to one molecule of superoxide dismutase which is a positive number of 1 or more).

The present invention also provides a treatment agent of diseases (hereinafter, sometimes referred to as the treatment agent of the present invention) comprised of the drug composition of the present invention.

The drug composition of the present invention has preferably the following characteristics:

- (a) property: when water for injection (distilled water for injection) is added to the lyophilized drug composition, it is dissolved and no insoluble foreign substances are observed;
- (b) stability: when the superoxide dismutase activity per unit weight immediately after lyophilizing this drug composition is set as 100, the relative values of the activity at the time points after the lyophilized composition has been stored at 8°C for 12 months, 25°C for 12 months, or 40°C for 6 months are all 97% or more;

(c) peaks of analogues in gel filtration chromatography: when the lyophilized composition is re-dissolved, submitted to gel filtration chromatography and absorbance of the eluates is measured at 220 nm, no substantial difference is observed between the peak shape of lecithin-modified superoxide dismutase on the detection chart of the absorbance and the peak shape of lecithin-modified superoxide dismutase before lyophilized; and

(d) peaks of analogues by reversed phase chromatography: when the lyophilized composition was re-dissolved at the time points after it has been stored at 8°C for 12 months, 25°C for 12 months, or 40°C for 6 months and submitted to reversed phase chromatography and absorbance of the eluates is measured at 220 nm and 270 nm, each amount of detected analogues is not substantially different from that immediately after lyophilized.

The term "dissolution" in this specification means that a solute (lyophilized drug composition) and a solvent (water for injection) are mixed to generate a macroscopically clear solution. For whether "dissolution" occurs or not, it is preferable to judge whether or not the solute can be dissolved within 10 seconds by adding the solvent (water for injection) to the solute at room temperature (1°C to 30°C) and shaking mildly.

Further, the drug composition of the present invention preferably maintains all characteristics described in the above described (a) to (d) even at any time points after the

lyophilized substance has been stored at 8°C for 36 months, 25°C for 36 months or 40°C for 6 months.

In addition, the present invention includes an agent having sucrose as an active ingredient for inhibiting (hereinafter, sometimes referred to as the inhibitor of the present invention) a reduction of the PC-SOD activity by making it coexist with PC-SOD represented by the above general formula (I), or for controlling appearances of peaks of analogues when analyzing the PC-SOD by column chromatography.

Furthermore, the present invention provides a method for inhibiting (hereinafter, sometimes referred to as the inhibitory method of the present invention) a reduction of the PC-SOD activity by making sucrose coexist with PC-SOD represented by the above general formula (I), or for inhibiting appearances of peaks of analogues when analyzing the PC-SOD by column chromatography.

The drug composition of the present invention is useful as a drug, for example, an ingredient of the treatment agent of the present invention, since reduction of the PCD-SOD activity due to long term storage is less (stability is higher) by an action of a drug carrier, particularly sucrose, in the composition, the property when lyophilized is good and appearances of peaks of analogues when analyzed by column chromatography are controlled.

The treatment agent of the present invention is useful as a drug, for example, a treatment agent for diseases caused by excess active oxygen, in which a reduction of the PCD-SOD

activity due to long term storage is less (stability is higher), the property when lyophilized is good and appearances of peaks of analogues when analyzed by column chromatography are less.

In addition, the present inventive inhibitor is useful when preparing the drug composition of the present invention and the treatment agent of the present invention, since it inhibits a reduction of the PCD-SOD activity due to long term storage (maintains stability), retains the property well when lyophilized and has an action of controlling appearances of peaks of analogues when analyzed by column chromatography.

Best Mode for Carrying Out the Invention

The Embodiment will be described below.

<1> The Present Inventive Drug Composition

(1) PC-SOD used for the drug composition of the present invention

The term "lecithin" in the present specification designates normal lecithin which means phosphatidylcholine, and "lysolicithin" designates a compound in which one molecule of fatty acid bonded at the 2-position of glycerol in lecithin is removed and a hydroxyl group is bonded to the carbon atom at the 2-position.

The PC-SOD contained in the drug composition of the present invention can be obtained usually by bonding one or more lecithin derivatives in which a chemical crosslinking agent is bonded to the hydroxyl group at the 2-position of

lysolecithin to SOD. The PC-SOD is represented by the following formula (I):



In the above described general formula (I), SOD' is a residue of superoxide dismutase.

As far as SOD' in the above general formula (I) can exhibit the essential function of decomposing active oxygen O_2^- in vivo, the origin and the like are not particularly limited to and SOD residues originated in different animals and plants or microorganisms may be widely used. However, considering that it is preferable to reduce antigenicity as much as possible in a living body as SOD for use of drugs, it is preferable to select suitably appropriate SOD according to animal types to which the drug composition of the present invention is administered. For example, when targeting humans which may most need the drug composition of the present invention, it is preferable to use human-derived SOD so as to reduce antigenicity as SOD in a human living body as much as possible. Among them, human-derived Cu/Zn SOD (human-derived SOD containing copper and zinc at the center of activity; hereinafter, sometimes abbreviated as human Cu/Zn SOD) is especially preferable because there is much amount of expression in cells, a production technique by a gene engineering method is established and the much amount of preparation is possible.

This human Cu/Zn SOD includes: natural human Cu/Zn SOD manufactured from human tissues; human Cu/Zn SOD manufactured

by a gene engineering method; recombinant human Cu/Zn SOD having substantially the same amino acid sequence as natural human Cu/Zn SOD; and SODs where partial amino acids in amino acid sequences of these human Cu/Zn SODs are modified or changed, and any human Cu/Zn SOD may be used.

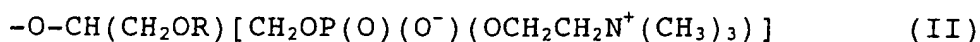
For reference, the amino acid sequence of this natural human Cu/Zn SOD is shown in sequence No. 1. In addition, the actual human Cu/Zn SOD is constituted as a dimer of protein having this amino acid sequence.

While as shown in the amino acid sequence of this protein, the amino acid at the 111-position in natural human Cu/Zn SOD is cysteine, there are also reported human Cu/Zn SOD where conversion to serine was performed at the 111-position by a protein engineering method, e.g. a site-directed mutagenesis (Japanese Patent Laid-Open No. 62-130684), and human Cu/Zn SOD where cysteine at the 111-position was chemically modified (Japanese Patent Laid-Open No. 6-199895), and the PC-SOD of the present invention can be obtained using these human Cu/Zn SODs as a raw material. Among these human Cu/Zn SODs, the PC-SOD of the present invention is preferably obtained using the human Cu/Zn SOD as a raw material where cysteine at the 111-position was chemically modified, e.g. the cysteine was modified to S-(2-hydroxyethylthiocystein) which is uniform in the electrical charge and molecular weight, stable for the SOD activity.

Thus, in the present specification, both those in which partial amino acids are substituted in the human Cu/Zn SOD

by a site-directed mutagenesis etc. and those obtained by chemically modifying partial amino acids of the human Cu/Zn SOD are simply designated as the human Cu/Zn SOD.

In addition, "a residue without a hydrogen atom of the hydroxyl group of lysolecithin having a hydroxyl group at the 2-position of glycerol" designated as B in the above described formula (I) is represented by the following formula (II):



(wherein R is a residue of fatty acid (acyl group)).

R is preferably a residue of saturated or unsaturated fatty acids of 10-28 carbons, more preferably a myristoyl group, a palmitoyl group, a stearyl group, an icosanoyl group, a docosanoyl group, and other residues of saturated fatty acids of 14-22 carbons, most preferably a palmitoyl group which is a residue of saturated fatty acid of 16 carbons.

Q in the above described formula (I) represents chemical crosslinking. The chemical crosslinking is not particularly limited if it can be chemically bonded (covalent bond) crosslinking SOD with lecithin. For the chemical crosslinking, residue-C(O)-(CH₂)_n-C(O)- is preferably exemplified. This residue is preferably a residue without hydroxyl groups at the both ends of the straight-chained dicarboxylic acid represented by formula HO-C(O)-(CH₂)_n-C(O)-OH, or a residue without parts corresponding to hydroxyl groups at the both ends of the dicarboxylic acid anhydride, the dicarboxylic acid ester, the dicarboxylic acid halide, or other reactive derivatives and

the like of the dicarboxylic acid. When Q in the above described formula (I) is a residue of the above described straight-chained dicarboxylic acid, one end of Q and B are linked through an ester bond. In such a case, the other end of the Q is considered to be directly bonded by an amide coupling etc. with an amino group of SOD. In such a case, SOD' shows a residue without one hydrogen atom of an amino group involved in the SOD bond. In the above described chemical crosslinking, n in group-(CH₂)_n- is an integer of 2 or more, preferably an integer of 2 to 10, most preferably 3.

m in the above described formula (I) represents the average number of bonds of lysolecithin to one molecule of SOD. m is one or more positive numbers, preferably 1-12 positive numbers, most preferably 4.

In manufacture of PC-SOD, coupling of a lecithin derivative to SOD can be performed according to the known method, e.g. the method described in Japanese Patent Laid-Open No. 6-54681. The manufacturing process of this PC-SOD is described in Examples.

It is preferable that the PC-SOD used in the drug composition of the present invention is purified to the extent that it is usable as a drug and substantially contains no substances which are not permitted as a drug.

For example, it is preferable to use the purified the PC-SOD having 2,500 U/mg (PC-SOD) or more specific activity, the purified one having 3,500 U/mg (PC-SOD) or more specific activity is more preferable. Herein, 1 U (unit) in the present

specification means an enzymatic amount of the PC-SOD inhibiting 50% of the reduction rate of NBT (nitro blue tetrazolium) measured by the method according to J. Biol. Chem. Vol. 244, No. 22, 6049-6055 (1969) using NBT under the condition of pH 7.8 at 30°C.

(2) Drug carrier used in the drug composition of the present invention

While a carrier used in the drug composition of the present invention is not particularly limited to if the drug composition fulfils all properties described in the aforementioned (a) to (d), sucrose is most preferable as regards of solubility and stability. It is preferable that sucrose used in the drug composition of the present invention is purified to the extent that it is usable as a drug, substantially contains no substances which are not permitted as a drug, and treated with activated charcoal. Treatment with activated charcoal can be performed by dissolving sucrose in water followed by bringing this aqueous solution into contact with activated charcoal. Such an activated charcoal treatment, for example, can reduce an endotoxin concentration in sucrose, further can inhibit appearances of peaks of analogues and can improve stability.

The endotoxin concentration in the carrier used in the drug composition of the present invention, especially sucrose is preferably 0.25 EU/mg or below, more preferably the detectable limit (0.006 EU/ml) or below.

While the endotoxin concentration, in the drug carrier, particularly in sucrose can be measured using the known measuring method of endotoxin, the Limulus test method using limulina/amebocyte/lysate components is preferable. As the Limulus reagent used in the Limulus test method, the endotoxin-specific Limulus reagent is preferably used. As the Limulus reagent, for example, the following commercially available Limulus reagents can be used; Toxicolor System LS-6, LS-20, LS-50M, Endospecy ES-6 and Endospecy ES-200 (Seikagaku Corporation).

(3) The drug composition of the present invention

The drug composition of the present invention comprises PC-SOD and a drug carrier, particularly sucrose. The composition is obtained preferably by formulating the PC-SOD described in (1) and the drug carrier described in (2), particularly sucrose. Thus, the drug composition of the present invention wherein: reduction of the PC-SOD activity due to long term storage is less (stability is higher); the property when lyophilized is excellent; and appearances of peaks of analogues when analyzed by column chromatography are controlled, can be obtained.

A mixing ratio of the PC-SOD to the drug carrier, particularly sucrose in the drug composition of the present invention can be suitably determined by a person skilled in the art according to the dosage mount, the form or the like. of the drug composition of the present invention, and the weight ratio of "PC-SOD/drug carrier, particularly sucrose" is

preferably about 0.1/100-80/100, more preferably 0.4/100-60/100, but not particularly limited to.

The fatty acid content in the drug composition of the present invention is preferably 0.13-0.15 $\mu\text{mol/mg}$ protein. The fatty acid content can be measured using, for example, YMC Co., Ltd.-made "a kit for analyzing long chained- or short chained-fatty acids".

As far as the enzymatic activity of PC-SOD and the effect of the present invention are not affected, components used usually for drugs such as other active ingredients of drugs, a conventional excipient, a binder, a lubricant, a coloring agent, a disintegrator, a buffering agent, an isotonicizing agent, a preservative and a soothing agent can be added to the drug composition of the present invention. For such components, substances which are physiologically acceptable, have purity to the extent that they are usable as a drug and substantially do not contain substances which are not permitted as a drug can be used.

Manufacture of the drug composition of the present invention can be performed using the PC-SOD and the drug carrier, particularly sucrose with the pharmaceutically known methods. The drug composition of the present invention is preferably in a solution, a frozen or a lyophilized form. The form of solution also includes the following modes of (1) and (2).
(1) Solution state before freezing and after thawing, in the drug composition of the present invention when frozen.

(2) Solution state before lyophilizing and after re-dissolving by adding a solvent, in the drug composition of the present invention when lyophilized.

Among them, the form of the drug composition of the present invention is preferably a lyophilized form. That is, the drug composition of the present invention is preferably the lyophilized drug composition.

Lyophilizing can be performed through suitably setting conditions etc. by a person skilled in the art according to the known lyophilizing method. For example, using a commercially available lyophilizer, this can be performed by setting so as to be carried out in order of the following steps (1) to (5). Herein, numbers in the parentheses shows time required for each step.

- (1) Cooling: for example, cool from a room temperature to -40°C to -50°C (about 0.3 to 1.5 hr).
- (2) Maintaining the cooling state while keeping a vacuum state: for example, maintaining -40°C to -50°C (12 hr or more).
- (3) Returning to a room temperature from the cooling state while keeping the vacuum state: for example, returning to a room temperature from -40°C to -50°C (about 3 to 15 hr).
- (4) Maintaining the room temperature while keeping the vacuum state (e.g. about 3 to 20 hr).
- (5) Returning to normal pressure.

In manufacture of the lyophilized drug composition composed of steps (1) to (5), time required for the above step

(2) should be preferably devised and the time is preferably 12 hrs or more.

For the lyophilized drug composition, the one which characteristics fulfill all conditions of the following (a)-(d) is particularly preferable.

(a) Property: when the lyophilized drug composition is observed macroscopically, the cake form of that is a tablet shape, and when water for injection is added to the lyophilized drug composition, it is rapidly dissolved and no insoluble foreign substances are observed (the property of the lyophilized drug composition is maintained well). Usually, it is dissolved in water for injection within 10 seconds.

(b) Stability: when the enzymatic activity per unit weight immediately after lyophilizing is set as 100 (%), the relative values (%) of the enzymatic activity at the time points after the lyophilized composition has been stored at 8°C for 12 months, 25°C for 12 months or 40°C for 6 months are 97% or more, preferably 99% or more. This stability can be measured by the method described in "(1-2-2) Stability test" in Example 1 explained later.

(c) Peaks of analogues by gel filtration chromatography: when absorbance is measured at 220 nm, no substantial difference is observed between the peak shape of PC-SOD (in the lyophilized drug composition) in the detection chart of the absorbance and the peak shape of PC-SOD before lyophilizing. The chromatography is preferably carried out by the method

described in "(1-2-3-1) Detection test for peaks of analogues by gel filtration chromatography" in Example 1 explained later.

(d) Peaks of analogues by reversed phase chromatography: after the lyophilized composition has been stored at 8°C for 12 months, 25°C for 12 months or 40°C for 6 months, the amount of analogues detected by measurement of absorbance at 220 nm and 270 nm is not changed substantially compared to that immediately after lyophilizing. The chromatography is preferably carried out by the method described in "(1-2-3-2) Detection test of peaks of analogues by reversed phase chromatography" in Example 1 explained later.

Further, the drug composition of the present invention preferably maintains all properties described in the above (a)-(d) also at any time points during the lyophilized composition has been stored at 8°C for 36 months, 25°C for 36 months or 40°C for 6 months.

The analogues described in the present invention comprise various substances generated by cleavage at various sites in mainly lecithin including liberated fatty acids etc. These analogues are considered to be generated by cleavage of lecithin linked to PC-SOD, thus, when much amount of lecithin linked to PC-SOD is cleaved, the function as PC-SOD is reduced, and when much amount of such analogues are contained, they are not preferable managerially for the quality, the standards, etc. as the drug.

While the drug composition of the present invention or further the one which are various forms of the composition

can be used as the final dosage form as it is for administration as a drug, they can be used as a raw material for a drug having other dosage forms, for example, a liquid preparation, a lyophilized preparation, etc.

The drug composition of the present invention is preferably used as a preparation for injection having PC-SOD as an active ingredient. For example, when providing the drug composition of the present invention as a preparation for injection of a solution state, the drug composition of the present invention of the solution state manufactured by the above described method can be filled up into an appropriate container such as an ampoule, a vial, and a syringe for injection and sealed, then it can be distributed itself or stored to be provided for administration as an injection.

When providing the drug composition of the present invention as a preparation for injection in a frozen state, the drug composition of the present invention in a freezing state can be retained in an appropriate container such as an ampoule, a vial, and a syringe for injection, etc. in a sealed state, then distributed or stored to be provided for administration as an injection by thawing before administration.

When providing the drug composition of the present invention as a preparation for injection in a lyophilized state, the drug composition of the present invention as a preparation for injection in a lyophilized state manufactured by the above described method can be retained in an appropriate container

such as an ampoule, a vial, and a syringe for injection, etc. in a sealed state, then distributed or stored to be provided for administration as an injection by dissolving it with distilled water for injection before administration. The drug composition of the present invention in the lyophilized state may be provided as a set with a solvent for dissolution.

The lyophilized state as a preparation for injection is preferable. That is, it is particularly preferable that the drug composition of the present invention is a form of the lyophilized composition for injection.

<2> The Present Inventive Treatment Agent

The treatment agent of the present invention is a treatment agent for diseases comprising the drug composition of the present invention. Further, the treatment agent of the present invention includes a concept such as a therapeutic agent, a preventive agent, an inhibitor for deterioration, an improving agent, etc. for different diseases. For PC-SOD, sucrose, these blending amount, other components which may be contained, a dosage form, etc. used as constitutive components of the treatment agent of the present invention, the same modes as the above described drug composition of the present invention can be adopted.

Diseases to which the treatment agent of the present invention is applied are not particularly limited to, as far as the diseases can be treated (treatment, prevention, maintenance (inhibition for deterioration), reduction (improvement of symptoms) and the like) by the action of PC-SOD.

The diseases treatable by the action of PC-SOD include those caused by excess reactive oxygen, more specifically include: motor neuron diseases (e.g. amyotrophic lateral sclerosis (ALS), spinal progressive muscle atrophy, familial spastic paraplegia, Charcot-Marie-leg, progressive bulbar palsy, juvenile-lateral upper limb muscle atrophy (Hirayama disease), etc.), ischemic reperfusion disorder, acute renal failures (prerenal acute renal failure, renal acute renal failure, postrenal acute renal failure, etc.), lupus nephritis (normal glomerular lupus nephritis, lupus nephritis accompanying mesangial proliferative change, focal segmental glomerulonephritis, diffuse proliferative glomerulonephritis, diffuse membranous glomerulonephritis, terminal sclerosing glomerulonephritis, etc.), functional disorders (allolalia, motor function disorder by paralysis, visual disorder, hearing disorder, smell disturbance, sensory disturbance such as dysgeusia, mental deterioration, etc.) accompanying cerebrovascular disorder (encephalorrhagy, hypertensive cerebral hemorrhage, cerebral infarction, cerebral thrombosis, brain embolism, transient ischemic attack, hypertensive encephalopathy, etc.), interstitial pneumonia, fibrosis (lung fibrosis etc.), ulcerative gastrointestinal injuries (ulcerative colitis, Crohn's disease, etc.), arthritis, heat injury, etc. Among them, motor neuron diseases (particularly ALS) and ulcerative gastrointestinal injuries (ulcerative colitis and Crohn's disease) are preferable indications. In addition, fibrosis

(particularly lung fibrosis) and interstitial pneumonia are also preferable indications.

The treatment agent of the present invention has an improving action to these diseases and to symptoms accompanying these diseases by an action of PC-SOD contained. Herein, the listed disease names are exemplified, but the application scope of the treatment agent of the present invention is not limited to such exemplified diseases.

In the present invention, it is preferable that the dosage forms described later are appropriately selected as those of the treatment agent of the present invention according to the properties and progress stages of targeted diseases.

The treatment agent of the present invention can be administered orally or parenterally depending on administration methods such as injection (intramuscularly, subcutaneously, intracutaneously, intravenously, etc.), peroral use, inhalation, etc. and can be appropriately formulated according to the administration methods. As a dosage form, while there are an injection (solution, suspension, emulsion, solid medicine for dissolution at use, etc.), a tablet, a capsule, a granule, a powder, a solution, a lipoid agent, gel, external powder, spray, inhalation powder, suppository, etc., the injection is preferable, and it is more preferable to prepare an injection for intravenous administration.

An amount of the active ingredient (PC-SOD) in the treatment agent of the present invention and an administration

amount of the above described treatment agent are varied depending on a preparation method of the formulation, a dosage form, a disease type, an administration method, an administration form, purpose for use of the above described treatment agent, a specific symptom of a patient (disease degree), body weight of a patient, etc., but not particularly limited to, for example, 0.5-100 mg (1,500-300,000 U) a day per adult can be exemplified as the clinical amount. In addition, for the dosing intervals of the above formulation, about once a day is possible and administration divided into 3 or 4 times is also possible.

While the treatment agent of the present invention can be also prepared as the one administered to vertebrate animals capable of developing the above described diseases, particularly mammals, it is preferable to prepare the treatment agent for humans.

<3> The Present Inventive Inhibitor and the Present Inventive Inhibitory Method

As described above, by making sucrose coexist with PC-SOD, reduction of the PC-SOD activity can be inhibited or appearances of peaks of analogues when analyzing the PC-SOD by column chromatography can be controlled.

Accordingly, the present inventive inhibitor is an agent having sucrose as an effective ingredient to inhibit reduction of the PC-SOD activity or to control appearances of peaks of analogues when analyzing the PC-SOD by column chromatography by making sucrose coexist with PC-SOD represented by the above

described general formula (I). Preferably, the agent is used for both purposes of inhibiting reduction of the above described PC-SOD activity is inhibited and of controlling appearances of peaks of analogues when analyzing the above described PC-SOD by column chromatography.

The present inventive inhibitor further exhibits an effect of retaining the property of the lyophilized drug composition well in addition to the above inhibitory effects by being made to coexist with PC-SOD when preparing the lyophilized drug composition. Thus, the present inventive inhibitor is preferably used by being made to coexist with the above described PC-SOD particularly when preparing the lyophilized drug composition containing the above described PC-SOD.

Further, the inhibitory method of the present invention is the one by which reduction of the PC-SOD activity is inhibited or appearances of peaks of analogues when analyzing the PC-SOD by column chromatography are controlled by making sucrose coexist with PC-SOD represented by the above described general formula (I). Preferably, the inhibitory method of the present invention is used for both purposes of inhibiting reduction of the above described PC-SOD activity and of controlling appearances of peaks of analogues when analyzing the above described PC-SOD by column chromatography.

Sucrose used as an active ingredient of the present inventive inhibitor or used in the inhibitory method of the

present invention and its amount added into PC-SOD etc. are similar to those described in the above described <1>.

Further, inhibition of reduction of the PC-SOD activity, control of peaks of analogues when analyzing by column chromatography and good maintenance of the property of the lyophilized drug composition when used for preparation of the composition can be confirmed by the methods described in Examples explained later.

Examples

The present invention will be specifically described by the following Manufacturing Examples and Examples. However, the present inventive technical scope should not be limited to these.

[Manufacturing Example]

<1> Preparation of Human Cu/Zn SOD

The SOD used is the one which was prepared according to the method described in Japanese Patent Laid-Open No. 6-199895. The amino acid at the 111-position of this human Cu/Zn SOD is S-(2-hydroxyethylthio) cysteine.

<2> Preparation of PC-SOD

PC-SOD was prepared by preparing 2-(4-hydroxycarbonylbutyryl) lysolecithin according to the method described in Japanese Patent Laid-Open No. 6-54681 followed by reacting this with the SOD of the above described <1>. After purification/concentration, the number of bonds of the lecithin derivative per molecule of SOD was obtained

by analyzing the protein concentration in according to the Lowry method (Lowry, O. H. et al., J. Biol. Chem., vol. 193, 265 (1951)) and by analyzing a residual amino group of SOD in according to the TNBS method (trinitrobenzene sulfonate, Goodwin, J. F. et al., Clin. Chem. vol. 16, 24 (1970)), to find that the average number of bonds was 4.0. The aqueous solution of this PC-SOD showed blue-green to green and pH showed 7-8. In addition, when examining the molecular weight by SDS-polyacrylamide gel electrophoresis, it was about 18,000 per monomer of the PC-SOD subunit (PC-SOD is a homodimer of the subunit).

[Example 1]

"PEG 4000" used in the present Example means polyethylene glycol (average molecular weight 4000).

(1) The methods used in this Example will be described together as follows.

(1-1) Lyophilizing

A solution composition was lyophilized using the lyophilizer (Model No. RL402BS; Kyowa Vacuum-made). The program of lyophilizing was set as performed in order of steps (1) to (5) in the following Table 1.

Table 1

Step	Pressure	Temperature	Time Required
(1)	Cooling	25°C → -40°C	0.5 hours
(2)	Maintaining vacuum state	maintaining at -40°C	12 hours

(3)	Maintaining vacuum state	-40°C → 25°C	12 hours
(4)	Maintaining vacuum state	maintaining at 25°C	16.5 hours
(5)	Returning pressure by nitrogen gas	maintaining at 25°C	several seconds

(1-2) Different test methods

(1-2-1) Property test

For the lyophilized composition, the property of the composition was examined by the following method.

Those where the cake form of the lyophilized composition was macroscopically tablet-like, and when added water for injection to the lyophilized composition, it dissolved within 10 seconds and there were no insoluble foreign substances observed were evaluated as ";", while those where the cake form of the lyophilized composition was not tablet-like or when added water for injection to the lyophilized composition, it did not dissolve within 10 seconds or there were insoluble foreign substances observed were evaluated as "x".

(1-2-2) Stability test

For the lyophilized composition, the enzymatic activity of PC-SOD in the composition was measured by the following method.

Using a solution obtained by adding water for injection to the lyophilized composition for reconstitution and using NBT under the condition of pH 7.8 at 30°C, the activity was measured by the method according to J. Biol. Chem. Vol. 244, No. 22, 6049-6055 (1969) to obtain the enzymatic amount

inhibiting 50% of the reduction rate of NBT as 1 U. Compared to the enzymatic activity immediately after lyophilizing set as 100 (%), the relative value (%) of the enzymatic activity after storage was calculated.

(1-2-3) Detection test for peaks of analogues by column chromatography

(1-2-3-1) Detection test for peaks of analogues by gel filtration chromatography (hereinafter, sometimes abbreviated as "GPC")

A solution obtained by adding water for injection to the lyophilized composition for re-dissolution was used as a sample, which was submitted to high performance liquid chromatography using the following column and mobile phase:

Column: YMC-Pack Diol-200 (YMC Co., Ltd.-made), and
Mobile phase: 45% acetonitrile/0.01M phosphate buffer.

The absorbance of the eluates at 220 nm was continuously detected to obtain the detection chart. The cases where no substantial difference was observed between peak shapes of PC-SOD on the detection chart and the peak shapes of the PC-SOD before lyophilizing were evaluated as ";", while the cases where the peaks not seen in those of PC-SOD before lyophilizing were observed were evaluated as "x".

(1-2-3-2) Detection test of peaks of analogues by reversed phase chromatography (hereinafter, sometimes abbreviated as "CN")

The sample was prepared similarly to the above (1-2-3-1) and submitted to high performance liquid chromatography using the following column and mobile phase:

Column: cyanopropylated silica gel (YMC Co., Ltd.-made), and

Mobile phase: acetonitrile/0.1% trifluoroacetic acid (elution at concentration gradient of 20%-90% of acetonitrile).

The absorbance of the eluates at 220 nm and 270 nm was continuously detected. The total area (SI) of a peak group (peak group of analogues) appeared in the front of a PC-SOD peak (main peak) and the total area (ST) of the PC-SOD peak and SI were measured to calculate an amount (SI/ST; %) of analogues for each sample.

For Tables 7 and 8 described later, the relative values are shown when an amount of analogues immediately after lyophilizing in prescription 1 set as 1 for easy comparison.

(2) Test for PC-SOD-containing drug composition

(2-1) Influence 1 by types of ingredients in the composition

PC-SOD (0.4 mg/vial) manufactured in the above described manufacturing Example and different ingredients (numbers in parentheses in the Table show weight per vial) were dissolved in water for injection to manufacture the solution composition and subsequently this was lyophilized.

The lyophilized substance was submitted to the above described different tests and the results are shown in Table 2.

Those where even one of x was present in the evaluation items were evaluated as "x", while those where no x was present in the evaluation items at all were evaluated as "T".

Table 2

Ingredient	Property Test	GPC	Judgement
Alanine (20 mg)	;	x	x
Inositol (20 mg)	;	x	x
Mannitol (20 mg)	;	x	x
Sorbitol (20 mg)	x	;	x
Sucrose (20 mg)	;	;	T
Creatinine (10 mg)	x	x	x
Glycine (20 mg)	;	x	x
PEG4000 (0.5 mg)	;	x	x
Urea (20 mg)	;	x	x
None	x	x	x

From the results of Table 2, it was clarified that the property of the lyophilized drug composition became extremely good and appearances of peaks of analogues when analyzing by column chromatography (GPC) were controlled by formulating PC-SOD together with sucrose.

(2-2) Influence 1 by types of ingredients

PC-SOD manufactured in the above described manufacturing Example and different ingredients shown in Table 3 were dissolved in water for injection to manufacture the solution composition, subsequently 0.5 mL each was separately poured into a vial and this was lyophilized according to the above described method.

The weight in the Table shows the one contained in a vial and "-" means no addition.

Table 3

	PC-SOD	Sucrose	PEG4000	Sorbitol	Mannitol
Prescription 1	2 mg	100 mg	-	-	-
Prescription 2	2 mg	5 mg	10 mg	-	-
Prescription 3	2 mg	-	-	5 mg	10 mg
Prescription 4	2 mg	-	10 mg	5 mg	-

The lyophilized drug composition of the above described prescription 1 to 4 was submitted to the property test, stability test and the detection test for peaks of analogues by gel filtration chromatography, and the detection test for peaks of analogues by reversed phase chromatography at the following time points (a) to (d):

- (a) immediately after lyophilized (not stored),
- (b) the time point stored at 40°C for 3 months after lyophilized,
- (c) the time point stored at 25°C for 3 months after lyophilized, and
- (d) the time point stored at 8°C for 3 months after lyophilized (the time point stored for 6 months in the prescription 1).

The results of each test are shown in Tables 4 to 7.

- (1) Property test

Table 4

Prescription	(a) Immediately after lyophilized	(b) 40°C for 3 months	(c) 25°C for 3 months	(d) 8°C for 3 (6) months
1	;	;	;	;
2	;	;	;	;
3	;	;	;	;
4	;	;	;	;

Any prescription showed the good property.

(2) Stability test

Table 5

Prescription	(a) Immediately after lyophilized	(b) 40°C for 3 months	(c) 25°C for 3 months	(d) 8°C for 3 (6) months
1	100	100	110	102
2	100	97	114	110
3	100	114	103	102
4	100	99	94	91

The prescription containing sorbitol and polyethylene glycol (prescription 4) showed greater reduction of PC-SOD activity than that of other prescription.

(3) Detection test for peaks of analogues by gel filtration chromatography

Table 6

Prescription	(a) Immediately after lyophilized	(b) 40°C for 3 months	(c) 25°C for 3 months	(d) 8°C for 3 (6) months
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1	;	;	;	;
2	;	;	;	;
3	;	N.T.	N.T.	;
4	;	;	;	;

In Table 6, N. T. means no performance of test.

No peaks of analogues by gel filtration chromatography were detected in any prescription.

(4) Detection test for peaks of analogues by reversed phase chromatography (220 nm)

Table 7

Prescription	(a) Immediately after lyophilized	(b) 40°C for 3 months	(c) 25°C for 3 months	(d) 8°C for 3 (6) months
1	1	1.0	1.0	1.1
2	1.5	1.8	1.6	1.8
3	1.5	3.4	2.2	1.8
4	1.4	3.4	2.2	1.8

In the prescription containing sorbitol and mannitol (prescription 3) and in the prescription containing sorbitol and polyethylene glycol (prescription 4), the peak area of analogues by reversed phase chromatography was about 1.4 to 3.4 times that of the prescription containing sucrose (prescription 1).

In addition, the experiments similar to the above prescription using 0.4 mg or 10 mg for the content of PC-SOD were conducted, consequently the similar results were obtained.

From the above results, it was found that the lyophilized composition containing PC-SOD and sucrose has extremely advantageous effects that: (1) the property is good; (2) reduction of enzymatic activity due to long-term storage is significantly less (extremely stable); and (3) peaks of analogues by gel filtration chromatography and reversed phase chromatography are extremely less, compared to the other prescription.

[Example 2]

In order to examine whether or not the lyophilized composition containing PC-SOD and sucrose could resist against the longer-term storage, the following tests were performed.

The lyophilized composition containing PC-SOD was manufactured with the above prescription 1, subsequently different tests were conducted according to the same method as Example 1 at the following time points (e) to (h). For each prescription of the present tests, 3 lots were submitted to the tests, respectively:

- (e) immediately after lyophilized (not stored),
- (f) the time point when stored at 40°C for 6 months after lyophilized,
- (g) the time point when stored at 25°C for 12 months (g-2), 24 months (g-2) and 36 months (g-3) after lyophilized, and
- (h) the time point when stored at 8°C for 12 months (h-2), 24 months (h-2) and 36 months (h-3) after lyophilized.

The results of the tests are shown together in Table 8.

Table 8

Test	(e) Immediately after lyophilized	(f) 40°C 6 months	(g-1) 25°C 12 months	(g-2) 25°C 24 months	(g-3) 25°C 36 months	(h-1) 8°C 12 months	(h-2) 8°C 24 months	(h-3) 8°C 36 months
Property	;	;	;	;	;	;	;	;
Stability	100	102	106	111	112	105	110	105
GPC	;	;	;	;	;	;	;	;
CN	1.0	1.2	1.0	1.0	1.1	0.9	1.0	1.1
	1.0	1.3	1.0	0.6	1.1	0.9	0.8	1.1

(the upper row of the column CN shows the results of 220 nm and the lower row shows those of 270 nm)

The experiments similar to this prescription using 0.4 mg or 10 mg for the content of PC-SOD were conducted, consequently the similar results are shown in Tables 9 and 10, respectively.

Table 9

Test	(e) Immediately after lyophilized	(f) 40°C 6 months	(g-1) 25°C 12 months	(g-2) 25°C 24 months	(g-3) 25°C 36 months	(h-1) 8°C 12 months	(h-2) 8°C 24 months	(h-3) 8°C 36 months
Property	;	;	;	;	;	;	;	;
Stability	100	104	104	110	104	104	110	108
GPC	;	;	;	;	;	;	;	;
CN	1.0	1.0	0.8	0.9	1.0	0.7	0.9	1.1
	1.0	1.6	0.9	0.7	1.0	0.8	0.7	1.1

(the upper row of the column CN shows the results of 220 nm and the lower row shows those of 270 nm)

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Table 10

Test	(e) Immediately after lyophilized	(f) 40°C 6 months	(g-1) 25°C 12 months	(g-2) 25°C 24 months	(g-3) 25°C 36 months	(h-1) 8°C 12 months	(h-2) 8°C 24 months	(h-3) 8°C 36 months
Stability	100	101	104	108	110	104	104	105
GPC	;	;	;	;	;	;	;	;
CN	1.0	1.2	1.0	1.1	1.2	1.1	1.0	1.1
	1.0	1.1	0.9	0.8	1.1	0.9	0.8	1.1

(the upper row of the column CN shows the results of 220 nm and the lower row shows those of 270 nm)

[Example 3]

30 mg of PC-SOD manufactured in the above described manufacturing Example and 50 mg of sucrose were dissolved in water for injection to manufacture the solution composition, subsequently 1.5 mL each was separately poured into a vial to lyophilize them according to the above described method.

At the time point immediately after lyophilizing the above described composition and that when stored at 8°C for 9 months, different tests were performed according to the same methods as in Example 1 except for the measuring method of the specific activity. The specific activity was measured by the method according to J. Biol. Chem., Vol. 244, No. 22, 6049-6055 (1969) using a solution which was obtained by adding water for injection to the lyophilized composition for re-dissolution and using cytochrome C under the condition of pH 7.8 at 25°C to obtain the enzymatic amount inhibiting 50% of the reduction rate of cytochrome C set as 1 U. Compared to the enzymatic activity immediately after lyophilizing was set as 100 (%), the relative values (%) of the enzymatic activity after storage were calculated. The results of tests are shown in Table 11.

